7-109488

JP 07-109488

19) Japanese Patent Agency (JP)

12) Official bulletin of publicly disclosed patents (A)

11) Number of public disclosure of patent application 7-109488

43) Date of public disclosure April 25, 1995

51) Int. Cl.6	Identification symbol	Number of in-office registration	FI
C 11 D 3/386			
17/00			
C 12 N 9/10		9359-4 B	
//(C 12 N 9/10			
C 12 R 1:07)			

Requests for examination not yet

Number of claims 2 FD

(8 pages in total)

---

21) Number of application patent application 5-277789

22) Date of application October 8, 1993

71) Applicant 000216162

Amano Pharmaceutical Co., Ltd.

Aichi-ken, Nagoya-shi, Naka-ku, Nishiki 1 chome 2-7

71) Applicant 591138810

Yoshiyuki TAKASAKI

Miyasaki-ken, Miyasaki-shi, Tachi-bana(?)-dori-nishi 1 chome 5-30

72) Inventor Yoshiyuki TAKASAKI

Miyasaki-ken, Miyasaki-shi, Tachi-bana(?)-dori-nishi 1 chome 5-30

7-109488

54) [Title of the invention]

Detergent composition.

57) [Summary]

[Aim]

To offer a detergent composition that not only has a washing effect, but also a deodorizing effect.

Construction]

It pertains to a detergent composition that contains cyclodextrin glucanotransferase, and with more details, it pertains to a detergent composition that contains cyclodextrin glucanotransferase that is produced by Bacillus sp. and that is stable to alkali.

[What is claimed]

[Claim 1]

A detergent composition that contains cyclodextrin glucanotransferase.

[Claim 2]

The detergent composition that has been mentioned in claim 1, wherein the cyclodextringlucanotransferase is an enzyme that is stable to alkali and that is produced by Bacillus sp.

[Detailed description of the invention]

[Field of use for the industry]

This invention pertains to a detergent composition that contains cyclodextrin glucanotransferase (below CGTase), and with more details, it pertains to a detergent composition that contains CGTase that is stable to alkali.

[Existing technology]

It is well known that hitherto enzymes such as protease, amylase, cellulase, lipase, and oxidoreductase etc. are mixed in

detergent compositions (for instance patent publication 60-52197, patent publication 61-60198, patent publication 59-49279, patent disclosure 63-161087, patent disclosure 1-60693 etc.). Moreover, for washing of tableware, in general amylase, protease and lipase etc. are used.

In recent years, automatic dishwashers have become popular, and even in the general household, they have come to be used. As detergents for such automatic dishwashers, ones wherein, as enzymes, α-amylase against soiling by starch materials, whereof boiled rice that has firmly adhered to the tableware, is a typical example, and lipase against fat soiling have been mixed, and various surfactants, bleaching agents, and perfumes etc. have been mixed, have been composed.

[Problems that should be solved by the invention]

The existing detergents had problems such as the fact that the mixing of α-amylase as an enzyme that removes soiling by starch that firmly adheres to tableware was still insufficient, the fact that also for protection of the natural environment the use of perfumes should be reduced or that they should not be used at all, and the fact that it is necessary to obtain sufficient washing power.

[Means to solve the problems]

The present inventors carried out serious studies on the above mentioned problems, with the result that they found that these problems can be solved by admixture of CGTase to the detergent, and they achieved the completion of this invention. That is to say that this invention pertains to a detergent composition that contains CGTase.

The cyclodextrin that is produced by the action of CGTase on starch, is known to have actions such as encapsulation of various organic compounds, stabilization of instable materials, preservation of perfumes, deodorization of bad smelling materials, removal of bitterness(?), stimulatation of emulsification, and improvement of sudsing etc., and it is used in the fields of medicines and food. Methods wherein for instance cyclodextrins are mixed in detergents and the sudsing properties thereof are improved (patent disclosure 63-68520, patent disclosure 2-34693,

patent disclosure 3-172397), and wherein a lasting effect of perfumes is achieved (patent disclosure 1-185399) are known.

This invention has been performed, based on the finding that it is possible to produce cyclodextrins by allowing CGTase to act on starch materials that have adhered to tableware etc., that are the object of washing, and to achieve suppression of generation of bad odour and improvement of the washing results by the use of actions such as deodorization and stimulation of emulsification of soiling, with the said produced cyclodextrins.

CGTase is an enzyme that produces cyclodextrins by acting on starch, and it is classified with EC 2.4.1.19 as the enyzme number. Moreover, cyclodextrin is a general name for annular oligosaccharides that consist of 6, 7 ot 8 glucoses.

There are no special limitations for the origin of the CGTases that can be used in this invention. They are known to be produced by bacteria of the genus Bacillus, such as Bacillus macerans, Bacillus megaterium and Bacillus stearothermophilus, and bacteria such as Klebsiella pneumoniae. Or some basophilic micro-organisms, for instance basophilic Bacillus no. 38-2, produce CGTase with an optimum pH at pH 5.5 and pH 8.5, and produce β-cyclodextrin from starch. Details of the CGTase that is produced by these micro-organisms have been summarized in for instance Fermentation and Industry (Hakko to Kogyo) 36 (3), 176-183 (1978), Brewing (Jozo) 80 (7), 434-440 (1985), 'Basophilic micro-organisms' 101-110 (1982, issued by (Co.) Gakkai Shuppan Center (Academic publication center) etc.

Moreover, the present applicants screened in the wide nature to obtain CGTases that are better for use in this invention. The result thereof was that they observed that the CGTase that is produced by Bacillus sp. YT-1 that was isolated from soil, has an optimum pH in the vicinity of 6, is stable in a wide pH range of 5-12, has the property of producing cyclodextrins in a wide pH range of 4-12, and particularly at pH 5-10, has the property of alkali resistance with an activity of 90% or more of the activity at the optimum pH, and that it suppresses the production of α-cyclodextrin, and produces β-cyclodextrin and γ-cyclodextrin as the main products at pH 9 or higher.

Because thus the cyclodextrins that are produced by this enzyme under an alkalinity of pH 9 or higher have as main compo-

nents β-cyclodextrin and γ-cyclodextrin with a large cavity, it was observed that this CGTase demonstrates, in addition to decomposition and removal of soiling by food, such as starch, also a remarkable effect of masking such as deodorization by the produced cyclodextrins, if it is used in detergents, and that it is extremely useful as a detergent component, for improvement of the cleaning capacity by improvement of the sudsing properties and stimulation of the emulsification of soiling etc.

The strain Bacillus sp. YT-1 that is used as an example of the micro-organism in this invention, is one that has been selected from a large number of micro-organisms that had been isolated from the world of nature for the above mentioned aim. The microbiological properties of Bacillus sp. YT-1 are as mentioned below. Moreover, this strain has been deposited as FERM P-13877 in the laboratory for industrial technology of bio-engineering of the institute for industrial technology.

(1) Morphology: rod-shaped bacteria (width 0.8-1.0 μm, length 3.0-4.0 μm)
(2) Spores: cause mycelium to swell with an egg-shape. Diameter 0.9-1.2 μm, formed at the end or in the center of the mycelium.
(3) Gram dyeing: negative
(4) Motility: +
(5) Oxidase: +
(6) Katalase reaction: +
(7) Indole: -
(8) VP: -
(9) pH in VP medium: 7.8
(10) Lithmus milk reaction: -
(11) Methylene blue reduction: -
(12) Tyrosine decomposition: -
(13) Growth in table salt (5, 7 and 10%): -
(14) Decomposition of urea: -
(15) Utilization of citrate (Christensen): -
(16) OF reaction: -
(17) State with respect to oxygen: aerobic
(18) Decomposition of glucose: -
(19) Decomposition of esculin: +

(20) Sulfate reduction: ±

(21) MacConky(?) growth: -

(22) Decomposition of starch: +

(23) Gelatin: +

(24) Tween 80: -

(25) DNase: +

(26) Phenylalanine: -

(27) Egg yolk: -

(28) Casein: + (18 days)

(29) Temperature of growth (° C): 10-41 (optimum 32-36)

(30) pH of growth: 7.2-11.5 (optimum 8.0-9.0)

(31) Growth in table salt: 0-1

(32) Nutrient requirement:

biotin	±
nyacin	-
thymine	-
folic acid	-
tryptophan	-

(33) Production of acids from sugars (phenolred half-liquid medium, 21 days)

mannitol	+
saccharose	-
xylose	-
sorbitol	-
salicin	+
arabinose	-
glycerin	-
dulcitol	-
glucose	-
maltose	-
mannose	-
lactose	-

The above mentioned microbiological properties were identified, referring to (1) Bergey's Manual of Determinative Bacteriology, vol. 2 (1986), Williams & Wilkins, USA, (2) N.R. Smith, R.E. Gordon, F.E. Clark (1952) Aerobic Sporeforming bacteria, Agr. Monograph, and (3) R.E. Gordon, W.C. Haynes, C.H. Pang (1973) The Genus Bacillus, Agr. Handbook no. 427, United States department of Agriculture, Washington DC. This micro-organism

does not grow in 2% table salt, and also the other properties are extremely similar to Bacillus brevis, but since it decomposes starch and does not decompose tyrosine, it could not be identified as this bacterium. Since, by the utilization of sugars etc., it does of course not correspond to Bacillus alcalophilus, Bacillus firmus, Bacillus lentus and Bacillus circulans etc., this bacterium was christened Bacillus sp. YT-1.

The outlines of the enzymatic properties of the CGTase that is produced by this micro-organism are shown below.

(a) Action and substrate specificity: It synthetizes α-, β- and γ-cyclodextrin by decomposition of starch. The rate of production of these cyclodextrins at pH 7-8 is that they usually are produced with a rate of 20 to 30 : 60 to 70 : 10 to 15, but at pH 9 or higher, the production of α-cyclodextrin is suppressed, and β-cyclodextrin and γ-cyclodextrin are produced as the main components, and the production rate usually is 0 to 3 : 80 to 85 : 10 to 20.

(b) Action pH and optimum pH: the action pH is 4-12, and the optimum pH, when determined after 30 minutes at 40° C in a 1% soluble starch solution of 10 mM acetate buffer or phosphate buffer, is 6-7, and at pH 5-10, it shows an activity that is 90% of the maximum activity or more (see figure 1).

(c) Action temperature and optimum temperature: The maximum of the action temperature is 80° C, and the optimum temperature, when determined after 10 minutes reaction in a 1% soluble starch solution of 10 mM phosphate buffer (pH 10), is 65° C (see figure 2).

(d) pH stability: When treated 3 hours at 25° C in a 10 mM acetate buffer solution or phosphate buffer solution, it is stable at pH 5-10 (see figure 3).

(e) Heat stability: when heated during 10 minutes in a 10 mM phosphate buffer solution (pH 10), it is stable up to 50° C, but at 60° C, it is 80% inactivated. In the presence of 5 mM calcium ions, 90% or more is retained in case of 10 minutes heating at 60° C (see figure 4).

(f) Molecular weight: the molecular weight that is obtained with the acrylamide electrophoresis method is 43,000.

(g) Stabilization: it is heat stabilized in the presence of Ca^{2+}.

(h) Inhibitors: the activity is inhibited by the presence of Hg^{2+}, Ag^{+}, Cu^{2+}, Ni^{2+} and Fe^{2+} etc.

(i) Method of purification: by DEAE-cephalose column chromatography and sephadex gel chromatography of the 60% ammoniumsulfate precipitate from the culture supernatant, it can be purified to homogeneity in electrophoresis.

(j) Method of determination of the activity: a proper quantity of enzyme solution was added to 0.2 ml of a 0.1 M phosphate buffer solution (pH 11.0) that contained 2% soluble starch, the total quantity was supllemented to 0.4 ml with water, and this was reacted during 10 minutes at 50^{0} C. After the reaction, 2 ml iodine-potassiumiodide-HCl solution [(addition of water to (0.05 g I_2, 0.05 g KI, 10 ml 0.1 M HCl) to a total quantity of 100 ml], and this was left standing at room temperature during 15 minutes and thereafter colorimetry was executed at 660 nm. The quantity of enzyme that causes a 1% reduction of the colour in 1 minute under these circumstances is 1 unit.

In the past, many micro-organisms that produce cyclodextrins have been discovered. The kind of the produced cyclodextrins depends on the kind of micro-organism, and the enzyme of Bacillus macerans, for instance, produces relatively much α-cyclodextrin (α:β:γ=13.3:2.3:1). In the same way, some bacteria, for instance Bacillus megaterium, produce relatively much β-cyclodextrin (α:β:γ=1.0:7.0:1.0). Moreover, the CGTase that is produced by basophilic bacteria, for instance Bacillus no. 38-2, has its optimum pH as 5.5 and 8.5, and produces β-cyclodextrin [Hakko to Kogyo 37(2), 150-161 (1979)].

The CGTase that is produced by Bacillus sp. YT-1, as the bacterium that is shown as an example in this invention, is stable in a wide pH range of pH 5-10, and has an optimum pH at 6-7, and when it is reacted at pH 6-8, it usually produces 20-30% α-cyclodextrin, 60-70% β-cyclodextrin, and 10-15% γ-cyclodextrin. When it is reacted at pH 9 or higher, on the other hand, the production of α-cyclodextrin is suppressed, and β-cyclodextrin and γ-cyclodextrin with large cavities are produced as the main components. The ratio thereof is 0-3% α-cyclodextrin, 80-85% β-cyclodextrin, and 10-20% γ-cyclodextrin (see table 1). The maximum yield thereof, however, is almost the same, 50-60%.

In the production of the CGTase of this invention by the cul-

ture of this micro-organism, organic nitrogen sources such as soy bean scrap, corn steep liquor, meat extract pepton, milk casein and yeast extract, are used as nitrogen sources. Among them, soy bean protein, soy bean scrap (defatted soy beans) and corn steep liquor etc. are good nitrogen sources. Besides, if necessary, inorganic nitrogen sources such as ammoniumsulfate, ammoniumchloride, urea and ammoniumnitrate are used. As carbon sources usually starch or starch derivatives such as liquefied starch, soluble starch and dextrin are used.

In addition to the above mentioned nitrogen sources and carbon sources, phosphates, magnesium salts, sodium salts and potassium salts are used as supplementary raw materials. Particularly addition of phosphates, magnesium ions and manganese ions is effective, and as the phosphates, for instance K_2HPO_4 in the order of 0.05-0.3%, as the magnesium salt, for instance $MgSO_4.7\ H_2O$ in the order of 0.01-0.3%, and as the calcium salt, for instance $CaCl_2$ in the order of 0.01-0.1% are added. Since CGTase is produced outside the mycelium, the enzyme is recovered by removing the mycelium after the culture by filtration or centrifugal separation.

As the CGTase that is mixed in the detergent composition of this invention, the ones that have been described above can be used without special restrictions, and for instance CGTase that originates in Bacillus macerans (trade name Contizyme, product of Amano Pharm. Co.) and CGTase that originates in Bacillus stearothermophilus (trade name KCGT, product of Amano Pharm. Co.) can be used. Since it is more preferred that the quantity of β- or γ-cyclodextrin that is produced is high, because usually detergents are alkaline, and also because the produced cyclodextrins sufficiently demonstrate the encapsulating effect of bad odours etc., the CGTase that is produced by the strain Bacillus sp. YT-1 (FERM P-13877), that was isolated by the present inventors from the world of nature, is used.

Moreover, apart from the CGTase, that is an indispensable component in the detergent composition of this invention, optional components that do not damage the functions of the washing effect and the deodorzing effect that are the aim, can be added. As such components, carboxymethylcellulose as resoiling preventing agent, sodiumcarbonate, sodiumbicarbonate and borax

etc. as alkaline agents, sodiumpercarbonate and sodiumperborate etc. as bleaching agents, silicones etc. as deodorants, and ethanol, isopropanol and propyleneglycol etc. as putrefaction preventing agents in the case that the detergents are offered as liquid detergents, can be mentioned. Moreover, in the case that no starch material, that is the object of washing, is present, the detergent composition may also contain starch materials such as soluble starch.

Experiments on the resistance of CGTase in detergents with a high alkalinity were carried out with commercial detergents, for instance Mamaroyal, product of Lion Co. (trade name, contains 30% surfactant), at 40° or 50° C in a solution with pH 10 or pH 11, that contained, as the surfactant, for instance 450 ppm.

Experimental example 1.

200 ml of a medium that consisted of 2% (as solids) corn steep liquor, 0.2% K_2HPO_4, 0.2% $MgSO_4.7H_2O$, and $1x10^{-3}$ M $CaCl_2$ were brought in an erlenmeyer flask of 1 liter, and after 15 minutes sterilization at 121% C, Bacillus sp. YT-1 (FERM P-13877) was inoculated, and this was cultured during 3 days at 30° C in a shaken culture with 225 rpm. The CGTase activity of the supernatant that was obtained by centrifugal separation after the culture was 184 units/ml medium.

To this culture supernatant, ammoniumsulfate was added to 60% saturation, and after collection of the precipitate that was produced, and dialysis, it was supplied to a DEAE-cellosolve CL-68 column that had been buffered with 25 mM tris buffer solution (pH 7.0), and eluted with KCl with a concentration that changed linearly from 0 to 1 M. The CGTase fraction was collected, concentrated, and after dialysis, subsequently gel filtration with a sephadex G-150 column was carried out. The obtained purified enzyme had 2960 units/A_{280}, and from the viewpoint of electrophoresis, it was homogeneous.

Experimental example 2.

1.0 ml 0.4 M glycine-sodiumhydroxide buffer solution, 0.3 ml CGTase that had been prepared in examples of execution 1 and 2 (381 units) and 3 ml liquid detergent with twice the standard concentration of use [product of Lion Co., trade name Mamaroyal,

surfactant (sodium α-olefinsulfonate, aliphatic acid alkanolamide, polyoxyethylenealkylether etc.) content 30%] were added, supplemented to a total volume of 6 ml with water, adjusted at pH 5.0, 7.0 and 10, and left standing at 35^0 C. In the course of time, fixed quantities were sampled, and the residual activity was determined. The obtained results are shown in figure 5.

As is clear from the figure, this enzyme is stable towards surfactants, and keeps 50% or more of its activity even after 140 hours of contact. Moreover, this enzyme has a higher stability at pH 10 than at pH 5 and pH 7.

Below, further details of this invention are described in the concrete by examples of execution, but the range of this invention is not limited to these examples of execution.

[Examples of execution]

Example of execution 1.

Rice starch (ca. 30 mg) that had been made to a paste, was pasted on a glass plate, and after drying, it was brought in the liquid detergent that had been used in experimental example 2, with various concentrations of CGTase, and they were immersed at pH 10 and pH 11, and at 40^0 C and 50^0 C. After 15 minutes the supernatants were collected, and after adjustment of pH to 5.0, respectively 0.05 ml of a 100 times diluted commercial Bacillus α-amylase (Thermamyl 6L, produced and sold by Novo Co.) and Aspergillus niger glucoamylase were added, and reacted during 15 minutes at 60^0 C. The thus produced reducing sugars were quantitatively determined with the Somogyi-Nelson method. The obtained results are shown in figure 6 and figure 7.

Figure 6 is the case of treatment at pH 10, and figure 7 is the case of treatment at pH 11. The quantity of enzyme of the horizontal axis is the quantity of CGTase of this invention that is present (x 25 units), and the vertical axis is the relative value (%) of the quantity of starch that had been decomposed and solubilized. In these figures, -●- is the case of 50^0 C, and -o- is the case of 40^0 C. In both cases, starch was decomposed and solubilized (converted to dextrin) by the CGTase in the detergent.

Example of execution 2.

Detergent compositions were obtained by the use of a composition as mentioned below and a commercial liquid detergent (experimental example 2) as the detergent composition, and addition hereto of various kinds of CGTase (100 units/ml).

Detergent composition 1.

sodium alkylbenzenesulfate	13%
sodium pyrophosphate	9%
sodium xylenesulfonate	5%
lauric acid diethanolamide	4%
water	69%

Detergent composition 2.

Pluronic	17%
CMC	9%
sodium tripolyphosphate	22%
Glauber's salt	45%
alkylamide of palm oil fatty acids	7%

The above mentioned detergent compositions (0.2%) were used, and the washing effect was evaluated with an automatic dishwasher (product of Matsushita Electr.). As the soiled dishes, 5 dishes that had been obtained by applying 2 g boiled rice on a magnetic dish (25 cm), and leaving them 24 hours at room temperature and drying, were used in the experiments.

The evaluation of the washing power was carried out by the colouring reaction of the residues of the boiled rice with iodine. For comparison, the same experiments were also carried out in the case without CGTase addition and in the case with addition of α-amylase. The results are shown in table 1.

Table 1.

enzyme detergency detergent	detergent 1	detergent 2	commercial product
CGTase (experimental example 1)	95	90	100
CGTase (Contizyme ?)	90	90	95
CGTase (KCGT)	90	85	95
α-amylase	85	70	90
no additives	70	75	85

In the table, the evaluation of the washing effect is shown as the detergency that is calculated from the proportion of the surface area of the coloured moiety that was produced by the iodine-starch reaction, and the initally soiled surface area. As is clear from the table, the case wherein a detergent composi-

tion that contains CGTase was used, showed the same or a better effect as the case wherein α-amylase was used.

Example of execution 3.

5 soiled dishes were immersed in the detergent compositions (0.5%) of example of execution 2, and left therein for 5 hours, and washing effect and deodorizing effect were evaluated. For comparison, the same experiment was also carried out for the case wherein no CGTase had been added and the case wherein α-amylase had been added. The results are shown in table 2.

Table 2.

enzyme / detergent	detergency deter-gent 1	deter-gent 2	commercial product	deodorizing effect in case of detergent 1 and detergent 2
CGTase (experi-mental example 1)	80	80	95	almost no bad odour
CGTase (Contizyme ?)	80	85	90	almost no bad odour
CGTase (KCGT)	85	85	90	almost no bad odour
α-amylase	85	90	90	peculiar odour observed
no additives	70	50	80	peculiar odour observed

In the same way as in example of execution 2, the washing effect was better than with α-amylase. The deodorizing effect was found to be remarkable only in the case that CGTase was used, and because the generation of bad odour that accompanies the washing, can be suppressed, addition of perfumes etc. is not necessary.

[Results of the invention]

This invention pertains to a cyclodextrin glucanotransferase that is produced by Bacillus sp. bacteria, and that produces β-cyclodextrin and γ-cyclodextrin at pH 9 or higher, and to a detergent that contains it as a component, and by the said enzyme, soiling by starch materials is effectively removed, and by the cyclodextrin that is produced, the cleaning effect is raised and also a deodorizing effect is demonstrated.

[Brief description of the figures]

[Figure 1]

shows the optimum pH of the CGTase of this invention (1% soluble starch, in 0.1 M buffer solution, 30 minutes reaction at 50° C), and -●- is the case wherein an acetate buffer solution is used, and -o- is the case wherein a phosphate buffer solution (KH_2PO_4-Na_2HPO_4 or NaOH-Na_2HPO_4) is used.

[Figure 2]

shows the optimum temperature of the CGTase of this invention (1% soluble starch, in 10 mM phosphate buffer solution (pH 10.0) 30 minutes reaction).

[Figure 3]

shows the pH stability of the CGTase of this invention. -●- is the case wherein an acetate buffer solution is used, and -o- is the case wherein a phosphate buffer solution is used.

[Figure 4]

shows the temperature stability of the CGTase of this invention. -o- shows the case wherein no calcium ions are present, and -●- shows the case wherein 5 mM $CaCl_2$ is present.

[Figure 5]

shows the pH stability of the CGTase of this invention in a detergent. It was left standing at 35° C at pH 5, 7 or 10. In this figure, -o- shows the case of pH 10, -□- shows the case of pH 5, and -●- shows the case of pH 7.

[Figure 6]

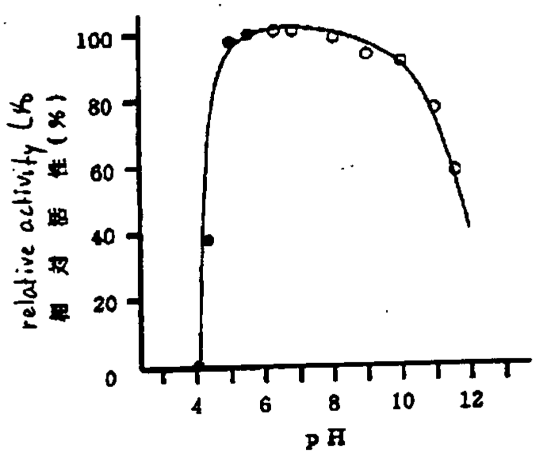
shows the action of decomposition and solubilization in the case that starch soiling is treated at pH 10 with the CGTase of this invention that has been added to a detergent. In this figure -●- shows the case of 50° C, and -o- shows the case of 40° C.

[Figure 7]

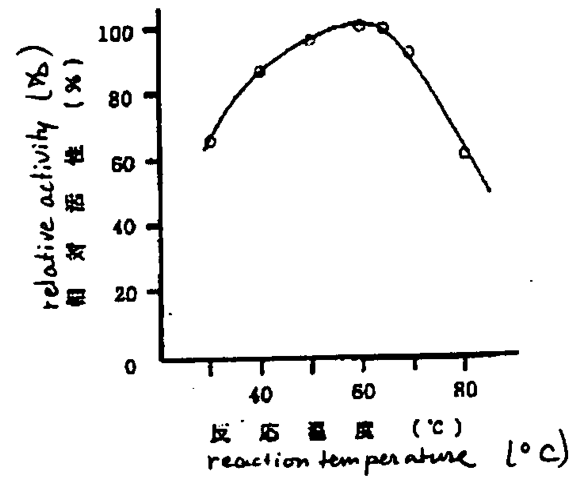
shows the action of decomposition and solubilization in the

case that starch soiling is treated at pH 11 with the CGTase of this invention that has been added to a detergent. In this figure -●- shows the case of 50° C, and -o- shows the case of 40° C.

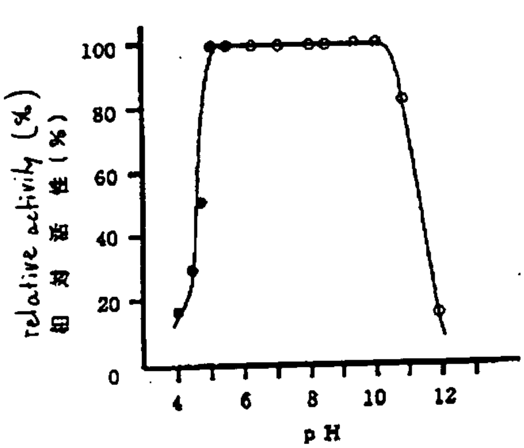
【図1】



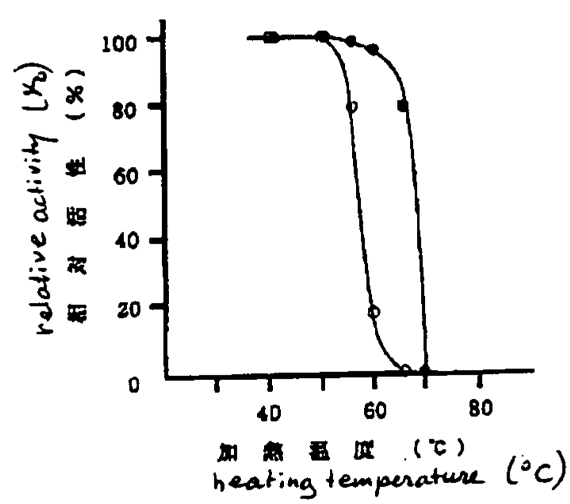
【図2】



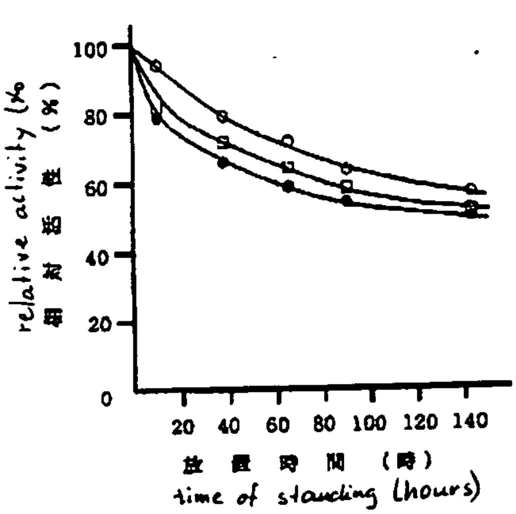
【図3】



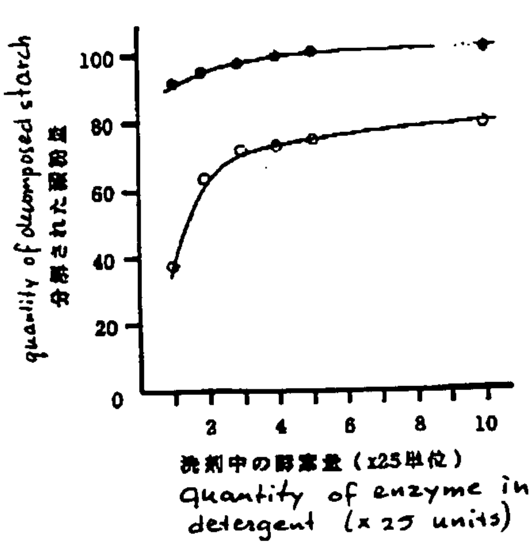
【図4】



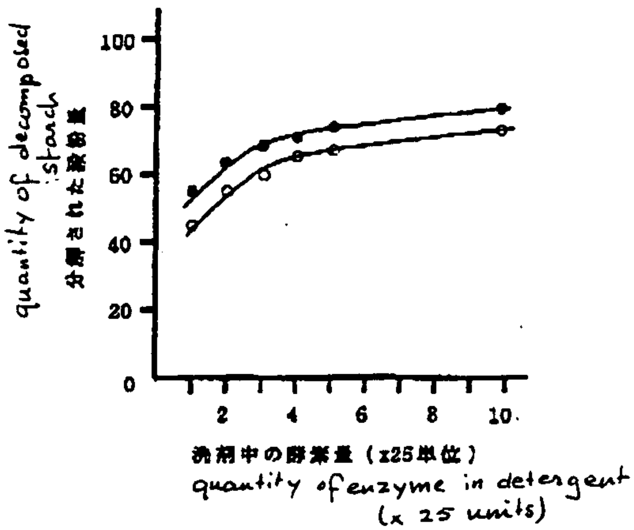
【図5】



【図6】



【図7】


100
80
60
40
20
0
2
4
6
8
10
分解された澱粉量
quantity of decomposed starch
洗剤中の酵素量（x25単位）
quantity of enzyme in detergent (x 25 units)